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(54) Title: METHODS OF TREATING DISEASE USING SERTOLI CELLS AND ALLOGRAFTS OR XENOGRAFTS (57) Abstract <p>The present invention describes a method of treating a disease that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. In particular, the present invention describes a method of treating diabetes mellitus by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site. A method of creating an immunologically privileged site in a mammal for cellular transplants is further described by the present invention. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is also provided.</p>			

**METHODS OF TREATING DISEASE USING
SERTOLI CELLS AND ALLOGRAFTS OR XENOGRAFTS**

This invention was made with United States government support under grant DK42421 awarded by the National Institutes of Health. The United States Government may have certain rights in the invention.

FIELD OF THE INVENTION

Transplants of healthy organs or cells into a patient suffering from a disease are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. The present invention provides a method of cellular transplantation in which an immunologically privileged site is created, thus alleviating the rejection associated with conventional transplantation therapy. Specifically, the present invention describes a method of treating a disease that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. In particular, the present invention describes a method of treating diabetes mellitus by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site. A method of creating an immunologically privileged site in a mammal for cellular transplants is further described by the present invention. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is also provided.

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BACKGROUND OF THE INVENTION

Certain chronic diseases destroy the functional cells in affected organs. Mammals with such diseases are often unable to produce proteins or hormones necessary to maintain homeostasis and usually require numerous exogenous substances to survive. Transplanting healthy organs or cells into a mammal suffering from such a disease may be necessary to save the mammal's life. This type of therapy is generally regarded as a last alternative to curing an otherwise fatal condition. Such transplants, however, are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. Presently, the only recourse to combat this immune response is to administer chronic nonspecific immunosuppression agents. Unfortunately, this only trades the complications of one chronic disease with other complications caused by the immunosuppression agent.

One disease which scientists have attempted to treat with organ and/or cellular transplants but have had very limited success is diabetes mellitus. Diabetes mellitus is a prevalent degenerative disease in mammals. It is characterized by a relative or complete lack of insulin secretion by the beta cells within the islets of Langerhans of the pancreas or by defective insulin receptors.

This insulin deficiency prevents normal regulation of blood glucose levels and often leads to hyperglycemia and ketoacidosis. When administered to a mammal, insulin promotes glucose utilization, protein

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synthesis, formation and storage of neutral lipids and the growth of certain cell types.

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In the United States alone there are approximately 13 million diabetics. Of these, 2.6 million are insulin dependent diabetics. Drug & Market Dev., 4:210 (1994). Healthcare analysts estimate that diabetes costs \$92 billion a year resulting from medical costs and lost productivity.

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The various forms of diabetes have been organized into a series of categories developed by the National Diabetes Data Group of the National Institutes of Health. Type I diabetes in this classification scheme includes patients dependent upon insulin to prevent ketosis. This group of diabetics was previously called juvenile-onset diabetes, brittle diabetes or ketosis-prone diabetes. Type I diabetes is caused by an autoimmune reaction that causes complete destruction of beta cells.

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Type II diabetes is classified as adult-onset diabetics. The diabetic patient may or may not be insulin dependant. Type II diabetes can be caused by a number of factors. For most mammals with Type II diabetes, the beta islet cells are defective in the secretion of insulin.

25

There are many therapies currently used to treat diabetes, however, each has its limitations. The major problem confronting most patients with diabetes mellitus is that currently available therapies fail to prevent the complications of the disease process. The most common method of treating Type I diabetes in mammals is providing an endogenous source of insulin

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such as porcine, bovine or human insulin. Insulin injection therapy prevents severe hyperglycemia and ketoacidosis, but does not completely normalize blood glucose levels. This treatment further fails to prevent the complications of the disease process, including premature vascular deterioration. Premature vascular deterioration is the leading cause of morbidity among diabetic patients. Furthermore, complications resulting from long-term diabetes include renal failure, retinal deterioration, angina pectoris, arteriosclerosis, myocardial infarction and peripheral neuropathy.

A second method of treating diabetes is by transplanting the pancreas in conjunction with the administration of chronic nonspecific immunosuppression agents. This treatment is usually given to an individual who has advanced diabetes, such as an individual with kidney failure. Whole pancreas transplantation can be successfully done with a 75% one-year survival rate, but surgical transplantation of the pancreas is very difficult. Furthermore, since the entire organ must be donated, the only practicable source is a deceased donor. In addition, when cyclosporine, the most common immunosuppressive drug used for organ transplants, is administered in a dosage necessary to suppress the immune response, the drug inhibits pancreatic cell function. Furthermore, the steroids that are often administered with an organ transplant often cause the patient to become diabetic.

A third treatment involves transplanting islet of Langerhans cells into the diabetic patient. However, islet transplantation has been generally unsuccessful due

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to the aggressive immune rejection of islet grafts.
(Gray, 1991, Immunology Letters 29:153; Jung et al.,
1990, Seminars in Surgical Oncology 6:122). In

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particular, successful transplantation of isolated
pancreatic islet cells has been very difficult to
achieve due to the chronic administration of
immunosuppressive drugs required to prevent organ
rejection of the cells following transplantation. These
10 dosages of immunosuppressive drugs can cause increased
susceptibility to infection, hypertension, renal failure
and tumor growth. Furthermore, unlike most organ
transplants, islet cells must grow their own blood
supply following implantation in the host in order for
15 the cells to survive. Conventional transplantation
techniques do not provide the necessary factors to
stimulate the production of new blood vessels.

The present invention alleviates many of the
problems associated with the current therapies for
20 chronic diseases that destroy the functional cells of
vital organs. In particular, the present invention
solves the problems associated with the conventional
therapies for diabetes mellitus, by providing a method
of transplanting pancreatic islets cells into a diabetic
25 mammal, whereby the cellular transplants produce insulin
in the diabetic mammal. The present inventor has
previously demonstrated extended functional survival of
islet cells allografts and xenografts in the testis.
(Selawry et al., 1989, Diabetes 38:220.) It has been
30 surprisingly discovered in accordance with the present
invention that an immunologically privileged site can be
created in a mammal by transplanting Sertoli cells to a

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notesticular site in a mammal. The newly created immunologically privileged site allows the transplantation and survival of cells that produce biological factors useful in the treatment of diseases, especially diabetes.

5 SUMMARY OF THE INVENTION

The present invention relates to a method of treating a disease that results from a deficiency of a biological factor in a mammal which comprises administering Sertoli cells and cells that produce the biological factor. In a preferred embodiment, the biological factor is a
10 hormone.

In a more preferred embodiment, the disease is diabetes mellitus, the factor producing cells are pancreatic islet cells and the factor is insulin.

In a particularly preferred embodiment, the present invention provides a method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said
15 method comprises administering *xenografted* Sertoli cells and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said *xenografted* Sertoli cells are administered in an amount effective to create an immunologically privileged site.

Another preferred embodiment contemplates a method of treating diabetes mellitus in a
20 mammal wherein said method comprises administering xenografted Sertoli cells and a therapeutically effective amount of pancreatic islet of Langerhans cells to a diabetic mammal in need of such treatment wherein said xenografted Sertoli cells are administered in an amount effective to create an immunologically privileged site.

In yet another embodiment the cells that produce the biological factors are cells that have
25 been genetically engineered, for example by transformation with a nucleic acid that expresses the biological factor.

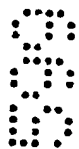
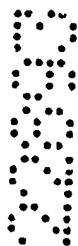
The present invention further relates to a method of treating diabetes mellitus in a mammal comprising administering pancreatic islet cells and Sertoli cells. In a preferred embodiment the Sertoli cells and islet cells are administered by transplantation.

30 Another aspect of this invention is directed to a method of creating an immunologically privileged site in a mammal.



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cryopreservation and thawing results in about 40% loss in number of islets in the absence of Sertoli cells and about a 20% loss in the presence of Sertoli cells; (3) cultured islets have the ability to respond to both glucose and glucose + Forskolin as insulin secretagogues; (4) the functional capacity of the co-cultured islet was enhanced two-fold in the presence of Sertoli cells;
5 (5) following cryopreservation and thawing, islets recover more rapidly in presence of Sertoli cells and the response to both glucose and glucose + Forskolin was enhanced two fold in the presence of Sertoli cells.



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Yet another embodiment of the present invention provides a pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor. In a preferred embodiment the pharmaceutical composition comprises Sertoli cells and pancreatic islet cells and a pharmaceutically acceptable carrier.

The present invention further provides a compartmentalized kit containing Sertoli cells and cells that produce a biological factor. An article of manufacture comprising a packaging material and Sertoli cells contained within the packaging is also provided.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the glucose responses to oral sustacal tolerance tests done on the monkey "Lucky" at intervals before pancreatectomy (Lucky-pre); after pancreatectomy but prior to transplantations (Lucky-post); and at intervals following transplantation (143 days, 730 days and 930 days, respectively).

Figure 2 shows the C-peptide responses to an oral sustacal tolerance test at the same time intervals as depicted in Figure 1.

25 Figure 3 shows the glucose responses to oral sustacal tolerance tests in the monkey "Oscar".

Figure 4 shows the C-peptide responses in the same animal and at the same intervals depicted for Figure 3.

30 Figure 5a and 5b show the effect of intratesticular islet allografts on serum glucose levels and the insulin responses to oral glucose in

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1 spontaneously diabetic BB/Wor dp rats. Figure 5a shows
the plasma glucose (mg/dl) concentrations in response to
the oral glucose administration of 2 g/kg of a 50%
glucose solution in three groups of rats: untreated
5 control Sprague Dawley, transplanted diabetic BB/Wor dp,
and insulin treated diabetic BB/Wor dp rats. Figure 5b
shows the serum insulin levels in response to the same
dose of oral glucose in untreated control Sprague
Dawley, and in transplanted BB/Wor dp rats.

10 Figures 6a and 6b show the effect of
intratesticular islet allografts on plasma glucagon
secretory responses to oral glucose and a combination of
glucose plus glipizide in spontaneously diabetic BB/Wor
dp rats. Figure 6a shows the plasma glucagon responses
15 to the oral administration of 2 g/kg of a 50% glucose
solution in three groups of rats: untreated control
Sprague Dawley, transplanted diabetic BB/Wor dp, and
insulin treated diabetic BB/Wor dp rats. Figure 6b
shows the plasma glucagon responses to the oral
20 administration of 7 mg/kg of glipizide and 2 g/kg of a
50% glucose solution, administered 30 minutes later, in
three groups of rats: untreated control Sprague Dawley,
transplanted diabetic BB/Wor dp, and insulin treated
diabetic BB/Wor dp rats. Data points are mean \pm SE of
25 eight animals in each group.

Figure 7 shows a light micrograph of the
pancreatic islets of Langerhans and the isolated rat
Sertoli cells transplanted into the renal subcapsular
space of a diabetic rat.

30 Figure 8 shows an electron micrograph of an
individual cell within the transplanted islet.

Figure 9 shows an electron micrograph of the
fine structure of the extra-islet cells labeled "S" in
Figure 7.

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1 Figure 10 shows the effect of transplantation
of piglet islets and Sertoli cells underneath the renal
capsule on the mean daily urine output of seven grafted
female rat recipients. Each bar represents the mean
5 daily urine output over a ten-day period following
transplantation.

 Figure 11 shows the effect of the
transplantation of piglet islets and Sertoli cells
underneath the skin on the mean daily urine volumes of
10 three rats over a 50-day period.

 Figure 12 shows the light photomicrograph of
pig islets of Langerhans and rat Sertoli cells
transplanted into the renal subcapsular space of a
diabetic rat. IL shows the presence of islands of beta
15 cells (IL) surrounded by an infiltration of small
lymphocytes underneath the renal capsule (K); B (upper
left) shows at higher magnification that the islands
(IL) consist of beta cells and B (lower right) shows
that beta cells contain characteristic insulin granules.

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DETAILED DESCRIPTION OF THE INVENTION

 The present invention is directed to a method
of treating a disease that results from a deficiency of
a biological factor in mammals which comprises
25 administering to a mammal Sertoli cells and cells that
produce the biological factor. As defined by the
present invention, a biological factor is a protein or
nonprotein compound that is necessary for cellular
metabolism and homeostasis. In a preferred embodiment,
30 the biological factor is a hormone. Hormone producing
cells which can be administered using the method
described in the present invention include, for example,

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1 pancreatic islet of Langerhans, pituitary, liver,
parathyroid and thyroid cells.

5 In accordance with the present invention, the
Sertoli cells and the cells that produce the biological
factor can be from the same species as the mammal to be
treated or from a different species. Further, the
Sertoli cells and the cells that produce the biological
factor need not be derived from the same species. It
has been demonstrated in accordance with the present
10 invention that Sertoli cells from pigs in conjunction
with islet of Langerhans from pigs can be used in the
treatment of diabetes mellitus in rats. In a preferred
embodiment the Sertoli cells are bovine, porcine or
human.

15 Sertoli cells, which are the predominant cells
of male testes, used in the method described by the
present invention can be separated from other testicular
cells such as Leydig cells, peritubular cells and germ
cells, using conventional techniques. For example, the
20 testes of a male mammal, such as a boar or ram, are
first collected by castration. The testes are then
chopped into several pieces and subsequently washed by
centrifugation.

25 Testicular Leydig cells can be removed from
the tissue suspension using digestion agents such as
trypsin and DNase. The remaining cell suspension is
then washed by centrifugation several times. The pellet
is resuspended in collagenase, incubated and washed by
centrifugation to eliminate peritubular cells within the
30 testes. Testicular germ cells can be removed by
incubating the pellet with hyaluronidase and DNase.
After several washings by centrifugation, the Sertoli

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1 cells can be collected to transplant using the method of
the present invention.

In accordance with the present invention, a
biological factor is a protein or nonprotein compound
5 that is absent, deficient or altered in a disease state.
Cells that produce a biological factor can be isolated,
for example, by first surgically removing the tissue
that produces the factor from a mammal. This tissue is
subsequently chopped and digested using conventional
10 techniques. For example, the tissue can be digested
using a collagenase digestion. The particular factor
producing cells can subsequently be collected from the
digestion mixture using a separation gradient such as a
Ficoll gradient. The factor producing cells are then
15 grown in tissue culture in serum using conventional
techniques. The factor producing cells may be co-
cultured with Sertoli cells in tissue culture. Cells
grown in tissue culture can be transplanted into a
mammal in conjunction with the Sertoli cells using the
20 method of the present invention. In accordance with the
present invention, factor producing cells may be stored
using a variety of conventional techniques, such as
cryopreserving the cells prior to growth in tissue
culture for subsequent transplantation. It has been
25 observed in accordance with the present invention, that
Sertoli cells co-cultured with factor producing cells
such as islet cells enhance the proliferation and
recovery rate of the factor producing cells in tissue
culture and in particular, enhance the recovery rate and
30 proliferation of factor producing cells that have been
previously stored using techniques such as
cryopreservation.

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1 In a preferred embodiment the factor is a
hormone, and the hormone producing cells are isolated
from a tissue source as described above. For example,
insulin-producing cells are isolated from the pancreas.
5 In another preferred embodiment, the factor producing
cells are provided by transforming suitable host cells
with a nucleic acid capable of expressing the factor of
interest. Transformed cells are provided by methods
known to one of ordinary skill in the art, and can be
10 found in a myriad of textbooks and laboratory mammals,
including Sambrook et al. (1989) Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor Laboratories, Cold
Spring, New York. If necessary, the nucleic acid
encoding the factor of interest can be adapted by
15 methods known to one of ordinary skill in the art to
effect secretion of the factor from the transformed
cell. The utilization of Sertoli cells in conjunction
with the factor producing cells in accordance with the
method of the present invention allows the production of
20 an immunologically privileged site in the treated
mammal.

The administration of factor producing cells
and Sertoli cells into a mammal is accomplished by
conventional techniques. In a preferred embodiment,
25 administration is by transplantation and the factor
producing cells are injected into the mammal
concurrently with or immediately after the injection of
the Sertoli cells into the same site. In accordance
with the present invention, an exogenous biological
30 factor may be administered following the transplantation
of factor producing cells and Sertoli cells until the
transplanted cells produce a therapeutically effective

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1 amount of the biological factor. For the treatment of
diabetes, for example, insulin may be administered
following the transplantation of pancreatic islet cells
and Sertoli cells until the transplanted islet cells
5 produce a therapeutically effective amount of insulin.

The Sertoli cells and factor producing cells
of the present invention can be transplanted using any
technique capable of introducing the cells into the
mammal such as parenteral administration or subcutaneous
10 administration following surgical exposure to a desired
site. Prior to transplantation, the recipient mammal is
anesthetized using local or general anesthesia according
to conventional technique. In a preferred embodiment
the mammal to be treated is human. In another
15 embodiment the present method of treating disease
further comprises administering an immunosuppressive
agent such as, for example, cyclosporine, tacrolimus,
despergualin and monoclonal antibodies to, e.g., T
cells. In a preferred embodiment the immunosuppressive
20 agent is cyclosporine. In another preferred embodiment
cyclosporine is administered at a dosage of from 0.5 mg
to 200 mg/kg body weight. In a most preferred
embodiment cyclosporine is administered at a dosage of
from 5 mg to 40 mg/kg body weight.

25 It has been discovered in accordance with the
present invention that administration of Sertoli cells
and factor producing cells results in the creation of an
immunologically privileged site in the treated mammal.
An immunologically privileged site as defined by the
30 present invention is a site in the mammal where the
immune response produced in response to the transplanted
cells is suppressed due to immuno-suppressive agents

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1 produced by Sertoli cells. Immunologically privileged
sites are characterized by an available blood supply to
provide nourishment for the transplanted cells and a
dense tissue to keep the transplanted cells within close
5 proximity of each other. Examples of immunologically
privileged sites as defined by the present invention
include the renal subcapsular space, subcutaneous facie,
the brain and the hepatic portal vein.

In accordance with the present invention it
10 has been shown that Sertoli cells increase the rate at
which the transplanted factor producing cells
vascularize in the transplanted site. It is therefore
indicated that the Sertoli cells (i.e. the relevant
agents produced by the Sertoli cells) promote the
15 increased vascularization rate of the transplanted
cells, for example, islet cells).

In a preferred embodiment, the present
invention describes a method of treating diabetes
mellitus by transplanting islet of Langerhans in
20 conjunction with Sertoli cells to create an
immunologically privileged site. Allografts as used in
the present invention describes the transfer of tissues
or cells between two genetically dissimilar mammals of
the same species. The term xenografts in the present
25 invention describes the transfer of tissues or cells
between two mammals of different species.

The transplanted islet of Langerhans cells and
Sertoli cells used in the method described by the
present invention can be prepared using any number of
30 conventional techniques. For example, islet of
Langerhans cells can be prepared from the pancreas of
several mammals of the same species. The pancreases are
pooled together, chopped up and digested using

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1 collagenase. The islet of Langerhans cells can be
further isolated using conventional gradients. Once
isolated, the islet cells can be grown in culture and
then transplanted in conjunction with Sertoli cells to
5 create an immunoprivileged site.

Sertoli cells used in the method described by
the present invention can be isolated from mammalian
male testes. To collect the islet cells, the testes are
first chopped into several pieces and then washed by
10 centrifugation. Leydig cells, present in the crude
mixture, can be removed from the tissue suspension using
digestion agents such as trypsin and DNase. The
remaining cell suspension is then washed by
centrifugation several times. Following, the pellet may
15 be resuspended in collagenase, incubated and washed by
centrifugation to eliminate peritubular cells within the
testes. Testicular germ cells can be removed by
incubating the pellet with hyaluronidase and DNase.
After several washings by centrifugation, the Sertoli
20 cells for transplantation can be collected.

The Sertoli cells can be transplanted to
create an immunoprivileged site within a mammal using a
variety of techniques. For example, after the mammal is
anesthetized, the Sertoli cells can be injected into a
25 tissue mass, thereby creating an immunoprivileged site.

Sertoli cells are administered in an amount
effective to provide an immunologically privileged site.
Such an effective amount is defined as that which
prevents immune rejection of the subsequently or co-
30 administered cells that produce the biological factor.
Immune rejection can be determined for example

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1 histologically, or by functional assessment of the
factor produced by the cells.

In a preferred embodiment Sertoli cells are
administered in amounts ranging from 10^1 to 10^{10} cells.

5 In a more preferred embodiment, 10^5 to 10^{10} cells are
administered.

The cells producing the biological factor are
administered in a therapeutically effective amount. The
ordinary skilled artisan can determine the appropriate
10 amount of cells producing the biological factor by
methods known in the art. The amount of cells is
dependent upon the amount of factor being produced by
the cells and the known therapeutically effective amount
of the factor necessary to treat the disease. For
15 example, 1 to 1000 islet cells per gram body weight can
be administered to treat diabetes using allografts, 20
to 1000 islets per gram body weight are administered
using xenografts. In another preferred embodiment, 5 to
100 islet cells per gram body weight are administered to
20 treat diabetes. In a most preferred embodiment, 5 to 20
islet cells per gram body weight are administered, using
allografts and 100-1000 islet cells per gram body weight
are administered for xenografts.

In another embodiment the present method of
25 treating diabetes further comprises administering an
immunosuppressive agent such as, for example,
cyclosporine, tacrolimus, desfergualin and monoclonal
antibodies to, e.g., T cells. In a preferred embodiment
the immunosuppressive agent is cyclosporine. In another
30 preferred embodiment cyclosporine is administered at a
dosage of from 0.5 mg to 200 mg/kg body weight. In a

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1 most preferred embodiment cyclosporine is administered
at a dosage of from 5 mg to 40 mg/kg body weight.

More generally, the immunosuppressive agent
can be administered for a time sufficient to permit the
5 transplanted islets to be functional. This period
extends from the point prior to or immediately following
the transplantation of the islets to the point at which
the cells are capable of producing therapeutically
effective amounts of insulin. In a preferred
10 embodiment, the sufficient period of time to administer
an immunosuppressive agent is about 40 to about 100 days
following transplantation of the islets. In a more
preferred embodiment, the sufficient period of time is
about 50-60 days.

15 A preferred embodiment of this invention is
directed to a method of treating Type I and Type II
diabetes mellitus by transplanting islet of Langerhans
in conjunction with Sertoli cells into the renal
subcapsular space.

20 Unlike the therapies for diabetes described in
the prior art, the method of treating diabetes described
by the present invention prevents the complications of
the disease process and does not result in the adverse
side effects associated with conventional diabetes
25 therapy. Furthermore, the method of transplanting islet
cells described by the present invention provides the
necessary factors for angiogenesis of the islet
transplants.

A method of creating an immunologically
30 privileged site in a mammal is further described by the
present invention. An immunologically privileged site
is created by transplanting isolated Sertoli cells into

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1 a mammal in an amount effective to create an
immunologically privileged site. In a preferred
embodiment, 10^1 to 10^{10} cells are administered. In a
more preferred embodiment, 10^5 to 10^{10} cells are
5 administered. In a preferred embodiment the Sertoli
cells are transplanted into the renal subcapsular space
or subcutaneous facie by injection. In a preferred
embodiment the mammal is a human and the Sertoli cells
are human or porcine.

10 Further contemplated in accordance with the
present invention is a method of enhancing the recovery
and proliferation of ex vivo cells comprising co-
culturing said cells with Sertoli cells for a time and
under conditions sufficient to achieve said enhanced
15 recovery and proliferation.

Another aspect of the present invention
provides a pharmaceutical composition comprising Sertoli
cells and cells producing a biological factor and a
pharmaceutically acceptable carrier. In a preferred
20 embodiment the composition comprises Sertoli cells and
islet of Langerhans cells and a pharmaceutically
acceptable carrier. A further preferred embodiment of
the present invention comprises using porcine, bovine or
human Sertoli cells and porcine, bovine or human islet
25 of Langerhans cells. As used herein, a pharmaceutically
acceptable carrier includes any and all solvents,
dispersion media, coatings, antibacterial and antifungal
agents, isotonic agents and the like. The use of such
media and agents is well-known in the art. The present
30 invention further contemplates a pharmaceutical
composition comprising Sertoli cells and a
pharmaceutically acceptable carrier.

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1 The present invention is also directed to a
kit for treatment of a disease. In one embodiment, the
kit is compartmentalized to receive a first container
adapted to contain Sertoli cells in an amount effective
5 to create an immunologically privileged site in a
mammal, and a second container adapted to contain a
therapeutically effective amount of cells that produce a
biological factor that is absent or defective in the
disease to be treated. In a preferred embodiment, the
10 Sertoli cells are bovine, porcine or human and are
provided in an amount of from 10^1 to 10^{10} cells. In a
more preferred embodiment, Sertoli cells are provided in
an amount of from 10^3 to 10^{10} cells. In another
preferred embodiment the cells that produce a biological
15 factor are cells that have been transformed with DNA
encoding the factor. In yet another preferred
embodiment the cells that produce the factor are
pancreatic islet cells. The islet cells are provided in
a preferred amount of 5 to 200 cells per gram of body
20 weight, and in a more preferred amount of 5 to 100 cells
per gram of body weight.

 The present invention further provides an
article of manufacture comprising a packaging material
and Sertoli cells contained within said packaging
25 material, wherein said Sertoli cells are effective for
creating an immunologically privileged site in a mammal,
and wherein said packaging material contains a label
that indicates that said Sertoli cells can be used for
creating an immunologically privileged site in a mammal.
30 The packaging material used to contain the Sertoli cells
can comprise glass, plastic, metal or any other suitably
inert material.

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1 In order to further illustrate the present
invention, the experiments described in the following
examples were carried out. It should be understood that
the invention is not limited to the specific examples or
5 the details described therein. The results obtained
from the experiments described in the examples are shown
in the accompanying figures and tables.

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EXAMPLE 1

5 Six male Rhesus monkeys were transplanted with islet allografts in their testes to examine the survival of these transplants. The recipients were made diabetic by means of a near total pancreatectomy, followed two weeks later by an intravenous injection of 35 mg streptozotocin/kg body weight. This procedure resulted in the induction of severe diabetes melitis. Plasma
10 glucose levels were in excess of 400 mg/dl and the animals were ketotic. Malabsorption was prevented by the oral administration of VIOKASE®, one tablet given twice daily before each meal.

15 Islets were isolated from female Rhesus monkeys. First, the pancreases of five animals were removed, pooled and chopped finely into smaller fragments. After collagenase digestion in a water bath at 37°C, the islets were separated from exocrine tissues and other cellular debris on at least two Ficoll
20 gradients, prepared in tandem. The islets were washed three times by centrifugation in ice-cold Hanks's buffer and then handpicked and transferred in groups of 150 to biologic grade Petri dishes. Each dish contained 6 mL of culture medium CMRL-1066 supplemented with 5% fetal
25 calf serum, glucose at a concentration of 250 mg/dL, penicillin (100 U/mL), and streptomycin (100 µg/mL). Incubation of islets were carried out at 35°C in 5% CO₂ and air for 4 to 6 days. The islets were transferred to fresh medium at 48 hour intervals.

30 Viability and counting of the islets were facilitated by means of the uptake of the dye dithizone. Each monkey received an average of about 10⁴ islets/kg

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1 body weight injected into both testes. In the first
three animals the testes were elevated into the
abdominal cavity, whereas in the last three recipients
the grafted organs were anchored into the inguinal
5 canal. Cyclosporine (CsA) was administered, in varying
doses to the first three grafted animals over a 30 day
period, whereas the last three hosts were given 7
injections of CsA (20 mg/kg) on days -4 to +3. Oral
sustacal tolerance tests were done on day 30, and then
10 at intervals in the normoglycemic animals, as follows.

The monkeys were housed individually in cages
and given standard monkey chow and fruit twice daily.
In addition, a pancreatic enzyme was mixed with the food
since the monkeys had been pancreatectomized to make
15 them diabetic before transplantation.

The night before the test, the animals were
fasted for 12 hours. At 8 a.m. the next morning they
were then anesthetized and prepared for the test meal.
Sustacal was used as the test agent. Sustacal consists
20 of a physiologic mixture of carbohydrates, proteins and
fat which closely mimics a standard meal and which is a
powerful stimulus for the release of insulin.

Sustacal was injected directly into the
stomach of the sleeping animal through a nasogastric
25 tube. Blood samples were then obtained at times 0, 15,
30, 60, 90, 120 and 180 minutes. The samples were
centrifuged and the serum stored at -20°C until
measurements for insulin or C-peptide could be carried
out. C-peptide is a very sensitive marker for beta cell
30 function. The results are shown in Figures 1-4.

Figure 1 shows the glucose responses to oral
sustacal tolerance tests done on the monkey "Lucky" at

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1 intervals before pancreatectomy (Lucky-pre); after
pancreatectomy but prior to transplantation (Lucky-
post); and at intervals following transplantation (143
days, 730 days and 930 days, respectively).

5 It can be readily appreciated that the animal
became severely diabetic after the removal of his
pancreas (Lucky-post). Following transplantation the
glucose responses were restored to normal levels at all
of the time intervals measured (143, 730 and 930 days
10 following transplantation). Lucky showed no evidence of
graft failure. With graft failure glucose levels would
become elevated would approach those which were found
following his pancreatectomy.

Figure 2 shows the C-peptide responses to an
15 oral sustacal tolerance test at the same time intervals
as depicted in Figure 1. Following his pancreatectomy
the C-peptide responses became blunted indicating a
severe diabetes. But following transplantation the
levels were not only restored to normal but appeared to
show a "hyperresponsive" pattern of C-peptide release
20 and levels done on day 730 exceed the normal levels at
all points measured. The elevated levels might be due
to the fact that insulin released from the testis enters
the systemic circulation. By contrast, insulin released
25 from the pancreas enters the portal vein and travels
immediately to the liver where about 60% is broken down
during the first passage. Insulin released into the
systemic circulation reaches the liver much later, thus
the elevated levels. As was evident with an
30 investigation of the glucose concentrations, the C-
peptide responses showed no evidence of failure 30
months following transplantation.

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1 Figure 3 shows the glucose responses to oral
sustacal tolerance tests in the monkey "Oscar".
Following the removal of his pancreas he became severely
diabetic with elevated glucose levels. Following
5 transplantation of islets the glucose responses became
similar to those determined before his pancreas was
removed. The glucose levels remain within normal levels
32 months following transplantation.

10 Figure 4 shows the C-peptide responses in the
same animal and at the same intervals depicted for
Figure 3. The animal became very diabetic following the
removal of his pancreas and shows blunted C-peptide
responses as a result. Following transplantation and
for the next 730 days the C-peptide responses were
15 greater compared with the normals. On day 930 following
transplantation the C-peptide responses have become
somewhat less compared with the normals. Despite
somewhat lower C-peptide levels the animal remains
normoglycemic.

20 This example demonstrates that primates can be
successfully transplanted with intratesticular islet
allografts without the need for sustained
immunosuppression, and that functional integrity of
intratesticular islet allografts is maintained for
25 periods exceeding two years with no evidence of graft
failure.

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EXAMPLE 2

This study examined insulin and glucagon secretory patterns in spontaneously diabetic bb/Wor dp rats transplanted with abdominal, intratesticular, islet grafts. Diabetic, BB/Wor dp, rats received intratesticular islet grafts from MHC-compatible BB/Wor dr rats and no immunosuppression. After a period of 74±15 days, of normoglycemia, three different groups (controls; BB/Wor dp, transplanted; and BB/Wor dp, insulin treated) were given the following challenges; (1) an oral glucose tolerance test (OGTT), (2) a single oral dose of glipizide, followed by an OGTT, and (3) arginine, by intravenous infusion. The results of this study are shown in Tables 1 and 2 and Figures 5 and 6.

TABLE 1

Metabolic Parameters and Immunoreactive Serum Insulin and Glucagon Levels in Control, and in Transplanted and Insulin treated, BB/Wor dp Rats.

	Controls	BB/Wor dp Grafted*	Insulin treated
Plasma Glucose (mg/dl):			
Prior to therapy	112 ± 5	502 ± 8+	510 ± 13+
After 2.5 months	97 ± 4	110 ± 3	350 ± 40 #
Duration p.t. OGTT (days)	75 ± 6	70 ± 11	78 ± 19
Weight gain (g)	120 ± 6	105 ± 17	48 ± 14 §
Fasting Plasma Insulin (uU/ml)	21.9 ± 3	20.4 ± 2	ND
Fasting Plasma Glucagon (pg/ml)	37.8 ± 5.7	43.4 ± 4.6	47.4 ± 4.9

* Duration of normoglycemia after grafting (days) = 279 ± 25; + P < 0.0001 vs. control
P < 0.0001 vs. grafted; § P < 0.02 vs. grafted

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TABLE 2

Pancreatic and Testicular Insulin and Glucagon Content in Control, and in Transplanted and Insulin Treated, BB/Wor dp, Rats.

	Controls	BB/Wor dp Grafted	Insulin treated
Pancreas (mg)	1573 ± 171	757 ± 122	920 ± 32
Insulin (ug/g)	66 ± 5.03	0.58 ± 0.18	0.76 ± 0.12
Glucagon (ng/mg)	4.1 ± 0.35*	4.9 ± 0.33**	6.9 ± 0.80
Testes fractions: (mg)	493 ± 49.6	582 ± 59.2	430 ± 28.0
Insulin (ug/g)	0.0	59.70 ± 0.49	0.0
Glucagon (ng/mg)	0.0	1.4 ± 0.37	0.0

* P < 0.03 and ** P < 0.08, vs. diabetic, respectively

Figure 5 shows the effect of intratesticular islet allografts on serum glucose and insulin responses to oral glucose in spontaneously diabetic BB/Wor dp rats. Figure 6 shows the effect of intratesticular islet allografts on plasma glucagon secretory responses to oral glucose and a combination of glucose plus glipizide in spontaneously diabetic BB/Wor dp rats. This experiment demonstrates that grafted testes in spontaneously diabetic BB/Wor dp rats contain both alpha and beta cells, and that the alpha and beta cells have the capacity to respond to specific secretagogues independently.

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EXAMPLE 3

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This study investigated the effect of Sertoli cell-enriched fraction (SEF) on islet allograft survival in the renal subcapsular space of diabetic rats.

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The animals used in this study were PVG rats, weighing between 150-200 g. Diabetes was induced by means of a single intravenous injection of 65 mg/dL of streptozotocin. Only rats with plasma glucose levels in excess of 400 mg/dL were transplanted. Sprague Dawley (S-D) outbred rats were used as islet donors. Either PVG or S-D male rats between 16 and 18 days old were used as Sertoli cell donors.

15

Islet Preparation

20

Islets were prepared according to modification of the method of London et al. (1990) Transplantation, 49: 1109-1113. The islets were purified on Ficoll gradients, and the isolated cells were then incubated for 4 days at 37°C in a humidified atmosphere of 5% CO₂ and air prior to use. No special efforts were made to deplete the islets of contaminating passenger leukocytes.

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Sertoli Cell-enriched Fraction Preparation

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Highly purified preparations of Sertoli cells were isolated from the testes of young males according to the method of Cheng et al. - J. Biol. Chem., 26:12768-12779. The testes were removed, chopped into several pieces, and placed in a 50 mL conical tube containing 50 mL of Ham's F12/DMEM media. The pieces were washed once by centrifugation at 800 x g for 2 min. The supernatant

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1 was aspirated, and the tissue resuspended in 40 mL of
media containing 40 mg trypsin and 0.8 mg DNase in a
sterile 250 mL Erlenmeyer flask. The flask was placed
in 37°C oscillating incubator at 60-90 osc/min for 30
5 min. This step removed Leydig cells. The tubules were
then transferred to a 50 mL conical tube, and
centrifuged at 800 x g for 2 min. The supernatant
fraction was aspirated, and the pellet resuspended in 40
mL of 1 M glycine, 2 mM EDTA containing 0.01% soy bean
10 trypsin inhibitor and 0.8 mg DNase, and incubated at
room temperature for 10 min. This step lysed any
residual Leydig cells. The cells were washed by
centrifugation for 2 min, and the step repeated twice,
or until the media was no longer cloudy. The pellet was
15 resuspended by gentle homogenization with a glass
Pasteur pipet in 40 mL of media containing 20 mg
collagenase in an Erlenmeyer flask, and incubated at
37°C for 5 min with 60-90 osc/min. The cell suspension
was centrifuged at 800 x g for two min, and the pellet
20 resuspended by gentle homogenization with a Pasteur
pipet in 40 mL media containing 40 mg collagenase and
0.2 mg DNase, and incubated in an Erlenmeyer flask at
37°C for 30 min with 60-90 osc/min. The cells were then
washed by centrifugation for 2 min, and the process
25 repeated at least three times to eliminate peritubular
cells. The cells were resuspended by gentle
homogenization with a Pasteur pipet in 40 mL media
containing 40 mg hyaluronidase and 0.2 mg of DNase, and
incubated at 37°C for 30 min with 60-90 osc/min. The
30 cells were pelleted by soft centrifugation for 2 min,
and washed at least five times to eliminate germ cells.
The resultant SEF was resuspended in 0.25 mL of media,

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1 and immediately transplanted into the recipient rat.
Each grafted rat received the equivalent of the total
amount of Sertoli cells contained in a single testis.

5 Transplantation of Rats

The diabetic rat was anesthetized with
methoxyflurane USP in a sterile hood and the left flank
opened to expose the kidney. The Sertoli-enriched
fraction containing approximately 5 million Sertoli
10 cells was injected first underneath the renal capsule.
The cells could be seen as a milkish bubble underneath
the capsule. Immediately afterwards, a total of 10
islets/g of body weight was injected to the same milkish
bubble. The needle was retracted slowly to prevent
15 leakage of the grafted cells. Cyclosporine (CsA) was
administered subcutaneously in varying doses over a 20-
day period to groups two and four. Because the grafted
rats responded similarly whether the drug was
administered over a 20-day, or over a 3-day period, all
20 of the subsequent groups, including the female rats,
were treated with only three injections of 25 mg/kg CsA,
given on days 0, +1, and +2, relative to the graft. The
rats received no other therapy.

A total of 36 male and 21 female PVG rats were
25 divided into six different treatment groups: Group 1,
the control group, consisted of 6 male rats grafted with
only islets from S-D donor rats. They received neither
SEF nor CsA. Group 2 consisted of 10 rats grafted with a
combination of islets from S-D rats and CsA
30 postransplantation, but no SEF. Group 3 consisted of a
total of 10 rats grafted with a combination of islets
from S-D and SEF from PVG donor rats, but no CsA

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1 postransplantation. Group 4 consisted of 10 rats
grafted with a combination of islets from S-D donors,
SEF from PVG donors, and CSA postransplantation. Group
5 consisted of 11 female rats grafted with the same
5 combination of cells as depicted for Group four. Group
6 consisted of 10 female rats grafted with a combination
of islets and SEF, both cell types from S-D donors, and
CSA postransplantation.

10 Postransplantation Evaluation of Rats

The grafted rats were transferred to metabolic cages, and plasma glucose levels were obtained at weekly intervals. Urine volumes and urine glucose contents were obtained at daily intervals. A rat was considered
15 cured of the diabetic process if the following criteria were met: A random plasma glucose level ≤ 150 mg/DL; glycosuria; and immediate reversal to hyperglycemia following surgical removal of the grafted kidney.

To determine if any of the rats had become
20 unresponsive to their grafts, normoglycemic rats were challenged with a secondary islet allograft consisting of at least 500, freshly prepared, Sprague Dawley islets which were injected into the contralateral renal subcapsular space. No immunosuppression was given
25 following the challenge.

To examine the impact of the transplantation of SEF on fertility of the female rats, normoglycemic animals of longer than 30 days were mated with PVG males. Metabolic parameters, as outlined above, were
30 closely monitored, as was the course of their pregnancies.

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1 Structural Analysis of Grafted Tissue

A total of five successfully grafted rats were nephrectomized at intervals following transplantation. Wedge sections of renal tissue, obtained from sites at which islets and SEF had been injected, were prepared for examination by light and electron microscopy, as previously described by Cameron et al. (1990) Transplantation, 50:649-653. Briefly, the tissue wedges were immersion-fixed with 5% glutaraldehyde in 0.1 M s-Collidine buffer for 1 h, washed in buffer, and post-fixed for 1 h with 1% osmium tetroxide in 0.1 M buffer. Small tissue blocks were cut from the wedges, and dehydrated through a graded series of ethyl alcohols, transferred to propylene oxide, and embedded in Epon 812/Araldite plastic resin. Thick (0.5 μ m) and thin (900 mg) sections were stained routinely with toluidine blue and uranyl acetate/lead citrate, respectively, for structural analysis by light and electron microscopy. The results are shown in Table 3 and Figs. 7-9.

TABLE 3

Effect of Sertoli cells on islet allograft survival in the non-immunologically privileged renal, subcapsular site

Group(n)	Gender	Sertoli cell (donor origin)	CaA	Duration of normoglycemia (days) Individual Responses
1 (6)	Male	--	-	0,0,0,0,0,0
2 (10)	Male	--	+	0,0,0,0,0,0,0,130 > 441, > 445
3 (10)	Male	+ (FVG)	-	0,0,0,0,9,10,12,13,13,14
4 (10)	Male	+ (FVG)	+	19,76,58*,84*,167*,127†,139†,>418†,>422†, >425†
5 (11)	Female	+ (FVG)	+	7,11,14,28,>287†,>305†,>306†,>308†,>441†,>447†,>457†
6 (10)	Female	+ (S-D)	+	8,10,96*,128*,>168,>172,>184,>193,>193,>196

* nephrectomized, †challenged with a secondary islet allograft.

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1 Group 1: None of the six rats grafted with islets alone, without either SEF or CsA, became normoglycemic.

5 Group 2: Three of 10 rats grafted with islets and treated with CsA became normoglycemic for more than 100 days. The 3 normoglycemic rats were challenged with a secondary graft on days 116, 192 and 197, respectively. One rat reverted to hyperglycemia on day 130, while 2 remained normoglycemic.

10 Group 3: Initially 6 of the 10 rats grafted with islets and SEF, but no CsA, became normoglycemic, but all of them reverted to hyperglycemia by day 14.

15 Group 4: All 10 of rats grafted with a combination of SEF and islets, and also given CsA became normoglycemic. Two reverted spontaneously to diabetes on days 19 and 76, respectively. Three were nephrectomized on days 58, 84 and 167 following transplantation. All 3 of these rats became hyperglycemic within the next 24 h. The remaining 5 rats were challenged with a secondary islet allograft on days 119, 129, 280, 342 and 400, respectively. Of these, the first 2 reverted to diabetes on day 127 and 139, respectively, while the latter 3 remained normoglycemic.

25 Group 5: All 11 of the female rats grafted with a combination of islets and SEF, and then given CsA, became normoglycemic. Of these, 4 reverted spontaneously to hyperglycemia by day 28. Of the 7 normoglycemic rats who were mated with male PVG rats, 6 became pregnant, and of these, 8 had litters varying between 1 and 10 pups. They were able to nurse the pups successfully. A total of 7 of the long-term surviving

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1 females were challenged with secondary islet allografts
at least 200 days following transplantation. None of
them reverted to hyperglycemia.

5 Group 6: Of the 10 rats grafted with islets
and SEF from the same donor strain of rat, all 10 became
normoglycemic. Two reverted to hyperglycemia by day 10.
A nephrectomy to remove the graft was done on 2 of the
long-term surviving rats on days 96 and 201,
respectively. Both reverted to hyperglycemic
10 immediately within the next 24 h.

Tissue Morphology

Renal tissue obtained from the long-term
grafted kidney appeared structurally normal by light
15 microscopy (Figure 7). Transplanted islets in this
organ were immediately subjacent to the kidney capsule,
and also appeared structurally normal. They displayed
tissue and cellular architecture identical to islets in
situ (Figure 7). Individual islet cells were
20 partitioned into cell clusters by thin connective septa
containing small vessels and capillaries (Figure 7). It
appeared that most of the islet cells contained
secretion granules. When resolved by electron
microscopy, islet cells were identified as the β -cell
25 type by the inclusion of ultrastructurally distinctive,
and unique insulin-containing secretion granules (Figure
8). All β -cell clusters observed were in close
proximity to intra-islet capillaries (Figure 8).

There was a high density of cells between, and
30 directly adjacent to, the transplanted islets and renal
parenchyma. By light microscopy, they did not appear to
be islet cells, kidney cells nor cells of blood origin

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1 (Figure 7). When observed by electron microscopy, these
cells were similar in ultrastructure to Sertoli cells in
that their nucleic were irregular in profile, and
5 contained deep nuclear clefts, distinctive nucleoli were
often present, and mitochondrial structure was dense.
Although these cells did not retain the typical polarity
of Sertoli cells in vivo, they were, however, identical
in appearance to Sertoli cells in vitro, when the cells
10 are not plated on a basement membrane substrate. The
cells were not associated with a basement membrane, and
appeared randomly organized (Figure 9). Cells showing
ultrastructural features of either germ or Leydig cells
were not observed.

15 This example demonstrates that an
immunologically privileged site for transplantation of
isolated islet can be created in male and female
diabetic recipients by transplantation of Sertoli cells
without the need for sustained immunosuppression.

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EXAMPLE 4

This study determined the survival of discordant islet xenografts in various nonimmunologically privileged organ sites in experimental animals.

Islets were prepared from young piglets as follows: Male piglets not weighing more than 2.2 kg were used exclusively. The piglet was anesthetized and following exsanguination both pancreas and testes were harvested under sterile conditions. A collagenase solution consisting of 2 mg/ml of collagenase type XI (Sigma) was injected directly into the pancreas. The pancreas was incubated at 37°C for 17 minutes and the digested tissues washed three times by means of centrifugation and aliquots of 1 ml each transferred to Petri dishes. The islets were incubated at 32°C in tissue culture media 199 supplemented with 10% horse serum for six days.

On day seven the cultured islets were collected in batches of 14,000 and cryopreserved using a standard protocol. The cells were stored in liquid nitrogen at -96°C for periods varying between two and four weeks. The islets were removed from the liquid nitrogen and thawed using an established procedure. The thawed islets were transferred to Petri dishes and co-cultured with pig Sertoli cells for three days at 32°C in the same 199 culture media as described above. Earlier studies have shown an improved survival rate of thawed islets cultured in the presence of Sertoli cells.

On day three following thawing the islets were hand-picked and counted and a total amount of 12 islets/g of body weight transplanted into female

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1 diabetic Sprague Dawley rats. A total of 5 million
Sertoli cells procured from the piglet testes were
grafted simultaneously into the same location. The
organ sites to be tested for the grafting of islets
5 include: a) the renal subcapsular space, b)
subcutaneously, and c) the liver. Following
transplantation, the rats were treated with cyclosporine
as follows: 25 mg/kg for 7 days; 15 mg/kg for 5 days;
10 mg/kg for 5 days; 5 mg/kg for an additional 13 days.
10 On day 30 the drug was discontinued.

To demonstrate viability and functional
integrity of isolated piglet islets the following
studies were done: a) staining of Cells with dithizone,
a stain which is highly specific for insulin; b)
15 staining of cells with 0.4% trypan blue which indicates
viability of the islets; and c) culturing of batches of
5 islets in the presence of insulin secretagogues such
as low and high glucose concentrations at specified
intervals following culturing, cryopreservation and
20 thawing. The results are shown in Table 4.

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TABLE 4

Insulin secretion (micro-units/ml) from incubated and from cryopreserved-thawed islets done on days 3, and 7, and 14, of culturing, respectively.

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Micro-units of Insulin release per 5 Islets

Days following isolation and incubation

3 Days 7 Days 14 Days

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Incubated islets prior to cryopreservation:			
a) Low glucose (90 mg/dl)	15.3 ± 3.8	21.8 ± 1.1	17.29 ± 2.4
b) High glucose (300 mg/dl)	32.2 ± 5.4	37.14 ± 3.4	23.3 ± 1.8
Cryopreserved and thawed islets			
a) Low glucose (90 mg/dl)	14.52 ± 2.8	7.13 ± 1.3	5.38 ± 2.02
b) Low glucose + Sertoli cells	10.31 ± 2.8	9.17 ± 2.6	8.38 ± .41

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TABLE 5

Yield of porcine islets following 1, 3, and 7 days of culture and the percentage of islets lost during 7 days of culture.

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Pig. No.	BW (kg)	Panc W g	D1 islets /g panc	D3 islets /g panc	D7 islets /g panc	islet loss % D7/D1
1	1.6	1.79	36,536	31,659	27,212	26%
2	2.0	1.89	37,272	32,962	27,883	25%
3	2.3	2.46	29,268	26,046	20,884	29%
4	1.8	1.66	39,904	37,726	31,664	21%
5	1.8	1.76	37,846	34,578	30,046	21%
6	1.6	1.74	39,866	37,888	32,424	19%
7	1.4	1.61	42,126	39,456	33,872	20%
8	2.3	2.48	33,682	29,334	24,892	26%
9	2.1	2.28	43,478	41,226	37,394	14%
10	2.1	2.09	40,126	36,448	33,282	17%
11	2.1	2.12	31,248	27,170	26,415	15%
12	2.1	1.98	38,848	36,465	29,293	25%
13	2.2	2.06	39,146	37,446	31,709	19%
14	2.2	2.24	27,892	25,028	21,342	23%
15	2.7	2.69	44,610	38,364	31,524	29%
16	1.5	1.44	42,222	40,414	31,244	26%
Mean \pm SE	2.0 \pm 0.3	2.0 \pm 0.4	37692 \pm 1233	34513 \pm 1307	29442 \pm 1119	22.2 \pm 1.2%

TABLE 6

Recovery of islets following freezing and thawing in presence and absence of Sertoli cells

Islets alone				Islets + Sertoli cells		
No. of islets	Pre-cryo	Post thawing	Recovery (%)	Pre-cryo	Post thawing	Recovery (%)
D3F/D3T	250	152	61%	290	212	73%
	230	131	57%	260	228	88%
	440	278	63%	430	380	88%
	420	366	87%	410	324	79%
	450	290	64%	440	358	81%
		Means	66.4%			81.8%
D7F/D3T	260	136	52%	250	229	92%
	300	208	69%	300	202	67%
	280	177	63%	290	238	82%
	360	205	57%	350	300	86%
	320	218	68%	390	289	74%
	380	217	57%	320	270	84%
		Means	61.0%			80.8%

As shown in Table 5, the yield of islets per gram pancreas was 37692 ± 1233 , 34513 ± 1307 and $29,442 \pm 1119$, after 1, 3 and 7 days of culture, respectively. Following cryopreservation and thawing and reculturing of the cells in the presence of Sertoli cells approximately 20% of the cells were damaged or lost as shown in Table 6. Thus $\pm 24,000$ islets/gram of piglet pancreas were available for transplant purposes after cryopreservation and thawing.

The results showed that insulin secretion was blunted when glucose was used as insulin secretagogue prior to cryopreservation. The effect was more evident following cryopreservation and thawing. While the

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1 presence of Sertoli cells had marked effects on number
of islets that survived cryopreservation and thawing
their presence had little effect on the ability of the
islets to respond to a low glucose concentration as
5 insulin releasing agent. However, as shown in Example 8
the presence of Sertoli cells augmented the secretion of
insulin in the presence of high glucose concentrations
and glucose plus Forskolin.

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EXAMPLE 5**Response of Diabetic Sprague
Dawley Rats to the Transplantation of
Islets from Piglet Donors (Discordant Xenografts)**

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The rats were made diabetic by means of a single i.v. injection of 55 mg/kg of streptozotocin. They were grafted only if the blood sugar was equal to or more than 400 mg/dl. Following transplantation the rats were placed individually in metabolic cages and urine volume, urine glucose content, and body weights were measured at daily intervals. Blood glucose levels were done at weekly intervals. A rat is considered cured of diabetes if the blood glucose level is 160 mg/dl or less and/or the daily urine volume is 15 ml or less.

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The results are illustrated in Figures 10 and 11.

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Figure 10 shows the effect of transplantation of piglet islets and Sertoli cells underneath the renal capsule on the mean daily urine output of seven grafted female rat recipients. Each bar represents the mean daily urine output over a ten-day period following transplantation. The study has been conducted over an 80-day period, the bar on the furthest right thus showing the mean urine output per day from day 80 through 89, etc.

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The figure shows that the mean daily urine volume for the first 60 days varied between 19.7 mls and 27 mls or within a diabetic range. It can be readily appreciated that urine volumes decreased to near-normal levels only from days 70 through day 89. The corresponding plasma glucose levels during the first and

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1 last then day periods were 474 ± 46 and 155 ± 70 , mg/dl,
respectively.

These results indicate that following
transplantation with piglet islets and Sertoli cells the
5 rats showed evidence of survival of the grafted islets.
The reversal to normoglycemia took about 80 days.

It should be noted that one of the cured rats
is pregnant and has been normoglycemic throughout her
pregnancy.

10 Figure 11 shows the effect of the
transplantation of piglet islets and Sertoli cells
underneath the skin on the mean daily urine volumes of
three rats over a 50-day period. The results show that
the mean urine volume decreased from a mean of 41.7 ml
15 during the first 10-day period to an average of 12.3 mls
during the fifth week. The corresponding glucose levels
were 509 ± 45 , and 200 ± 12 , mg/dl, respectively.

The data depicted above demonstrate that both
the renal subcapsular space and the subcutaneous area
20 can be used as a site to create an immunologically
privileged site for the transplantation of islet
xenografts.

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EXAMPLE 6

5 This study determined the effect of cultured Sertoli cells on the survival of discordant islet xenografts in diabetic rats with minimal early exogenous immunosuppression.

Preparation of Islets

10 Neonatal piglets of less than seven days of age were killed by anesthesia and islets were isolated according to a method of Kuo C.Y., Burghen G.A., Myvack A. and Herrod H.G. (1994) "Isolation of islets from neonatal pig pancreatic tissue", J. Tissue Culture Methods, 16:1-7. Briefly, the pancreas was distended by
15 an injection of a collagenase solution, 2 mg/ml, collagenase type X1, in culture medium DMEM. After incubation at 39°C for 17 min, the digested fragments were washed by centrifugation and the digested tissue was then incubated for one week in medium 199
20 supplemented with 10% horse serum and 1% antibiotics at 32°C. The islets were then cryopreserved according to the method by Lakey J.R.T., Warnock G.L., Kneteman N.M., Ao Z., Rajotte R.V. (1994) "Effects of pre-cryopreservation culture on human islet recovery and in vitro function", Transplant Proc., 26:820 and stored in
25 liquid nitrogen at -196°C. Three days prior to transplantation the cryopreserved islets were rapidly thawed and cultured at 32°C for two days. One day prior to transplantation some of the islets were collected and
30 co-cultured with Sertoli cells for 24 hours.

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1 Sertoli cell isolation

 Testes of young S-D rats were removed and Sertoli cells were isolated by the method of Cheng C.Y. and Bardin C.W. (1987) "Identification of two
5 testosterone-responsive proteins in Sertoli cell-enriched culture medium whose secretion is suppressed by cells of the intact seminiferous tubule." J. Biol. Chem., 262:12768-12779. Briefly, the testes were
10 digested first in DMEM containing 1.0% trypsin, and then is DMEM containing 1.0% collagenase, type 1, for periods of 15 min each, at 37°C. The purified Sertoli cells were cultured at 37°C in DMEM/F12 supplemented with transferrin, 10 ug/ml, FSH 10 ng/ml, insulin 20 ug/ml and 1.0% FCS, for three days. For transplantation,
15 Sertoli cells and islets were pooled and rats were grafted with either a composite consisting of 5×10^6 Sertoli cells and 3,000 islets, or with islets alone (15 islets/g of body weight).

20 Transplantation of rats

 Female S-D rats, weighing between 170 and 200 g were made diabetic by means of a single i.v. injection of 60 mg/kg of streptozotocin. A total of 31 diabetic rats were divided into 3 groups and grafted as follows:
25 Group 1, a control group (n=8), received a total of 15 islets/g body weight injected underneath the renal capsule. No Sertoli cells were grafted. Following transplantation the rats were treated with cyclosporine for 55 days: 25 mg/kg for 3 days, 15 mg/kg for 10 days,
30 10 mg/kg for 10 days and 5 mg/kg for the following 32 days. Immunosuppression was then stopped. Each rat received, in addition, 1-3 U of Ultralente insulin at

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1 daily intervals if the 24-hour urine glucose content
exceeded 1 g. Insulin therapy was stopped on day 55.
Group 1, a tissue control group (n=8), was given a
renal, subcapsular injection of a composite of about 5 x
5 10⁶ Sertoli cells and 3,000 islets. No CsA was given.
Insulin was given as depicted above. Group 3, the
experimental group (n=15), was transplanted with both
Sertoli cells and islets and then treated with CsA and
insulin according to the schedule outlined above.

10

Posttransplantation evaluation of rats

Plasma glucose levels were obtained at weekly
intervals. Twenty four hour urine volumes and urine
glucose contents were recorded daily. A rat was
15 considered cured of the diabetic process if the
following criteria applied: A plasma glucose level of
equal to or less than 10 mmol/L, a 24-hour urine volume
of less than 15 ml, and immediate reversal to
hyperglycemia following surgical removal of the grafted
20 kidney. One normoglycemic rat was mated on day 69 to
test her ability to become pregnant.

Structural analysis of the grafted tissue

Two normoglycemic rats were nephrectomized on
25 days 117 and 330 and grafted tissue prepared for light
and electron microscopy. Selawry H.P., Cameron D.F.
(1992) "Sertoli cell-enriched fractions in successful
islet cell-transplantation", Cell Trans., 2:123-129.
Briefly, tissue wedges were immersion-fixed with 5%
30 glutaraldehyde in 0.1 M collidine buffer for 1 h.,
washed in buffer, and postfixes for 1 h with 1% osmium
tetroxide in 0.1 M buffer. Small tissue blocks were cut

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1 from the wedges, and dehydrated through a graded series
 of ethyl alcohols, transferred to propylene oxide, and
 embedded in Epon 812/Araldite plastic resin. Thick (0.5
 5 um) and thin (900 ng) sections were stained routinely
 with toluidine blue and urinal acetate/lead citrate,
 respectively, for structural analysis by light and
 electron microscopy.

The results of the effect of Sertoli cells and
 cyclosporine on survival of xenographic transplantation
 10 of pig islet cells into the renal subcapsular space of
 diabetic female rats are shown in Table 7.

TABLE 7

15

Group (n)	Sertoli Cells	CsA	Graft Survival (days)
1 (8)	-	+	0,0,0,0,0,0,0,0
2 (8)	+	-	0,0,0,0,0,0,0,0
3 (15)	+	+	0,0,0,0,0, 71, 77, 96, 117*, 148#, >154, >165, >327, 330*

20

* rats nephrectomized to remove the xenograft

rat died during a cardiac puncture

As shown in Table 7, none of the rats grafted with
 islets alone and then given CsA and low-dose insulin
 25 (Group 1) became significantly less hyperglycemic.
 Further, none of the rats grafted with a composite of
 islets and Sertoli cells, but without CsA, showed any
 improvement of hyperglycemia (Group 2). Of 15 rats
 grafted with islets and Sertoli cells and then given CsA
 30 (Group 3), 10 showed evidence of reversal of the
 diabetic state. Four of the ten are still normoglycemic
 for periods of more than 154, 165, 165, and 327 days,

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1 respectively. The normoglycemic rats who were
nephrectomized on days 117 and 330, became hyperglycemic
immediately. Their plasma glucose levels were 4.9
mmol/L, and 8.2 mmol/L, prior to, and 20.7 mmol/L, and
5 32.2 mmol/L, respectively, following nephrectomy. A
female rat who was mated on day 69 became pregnant and
delivered a total of 10 pups on day 89, all of whom she
nursed successfully while remaining normoglycemic. She
died on day 148 as a result of a cardiac puncture.
10 Three of 10 rats regressed into hyperglycemia on days
71, 77, and 96, respectively, after a short period of
euglycemia.

These results demonstrate that prolonged survival
of a discordant islet xenograft (pig to rat) can be
15 achieved in female diabetic rats. Survival of islet
xenografts depended upon two factors which had to be
administered concomitantly: Co-transplantation with
Sertoli cells and treatment with cyclosporine.

The response of total urine volumes following
20 transplantation with a composite of pig islet and rat
Sertoli cells measured at 10-day intervals over an 80
day period for 7 of the improved rats showed an average
daily urine volume of 27.0 ± 13.0 ml/rat during the
first 10-day period, which slowly declined to a mean of
25 12.0 ± 4.0 ml/rat, 70 days following transplantation.

Tissue morphology studies shown in Figure 10 show
that the tissue and cellular structure of kidney
parenchyma appeared normal in the rat nephrectomized 117
days following transplantation. Normal appearing islets
30 with structurally distinct B-cells were visible in well
vascularized areas subjacent to the kidney capsule.
Additionally, normal appearing Sertoli cells were

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1 observed adjacent to the transplanted islets along with
numerous lymphocytes. No plasma cells were identified
at the transplantation site. Viable endocrine cells
were similarly observed in the subcapsular renal space
5 of the rat nephrectomized 330 days following
transplantation.

These studies show that significant prolongation of
survival of a discordant islet xenograft can be achieved
without sustained immunosuppression. These studies
10 demonstrate that the mechanism by which Sertoli cells
promote islet xenograft survival is three-fold: (1)
Sertoli cells stimulate the recovery of islets damaged
during transplantation (i.e. improve the yield and
function of cultured islets), (2) Sertoli cells protect
15 grafted islets from immunologic rejection by producing
factors which strongly suppress proliferation of T-
cells, and (3) Sertoli cells protect grafted islets from
the toxic effects of cyclosporine.

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EXAMPLE 7

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This study shows a method of isolating and cryopreserving porcine pancreatic islets for future xenographic transplants in mammals.

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Male piglets, < 7 days old and weighing $2 \pm$ kg were used as donors. The pancreases, weighing 1.4 ± 0.3 g, were harvested and injected with DMEM solution containing 2 mg/ml collagenase XI. The distended pancreas was incubated in a shaking water bath at 39°C for 17 min. The digested tissue was filtered through a $500 \mu\text{m}$ stainless steel filter and filtrates were washed x 3 with cold DMEM. Without further purification the cells were cultured in M199 and 10% horse serum at 32°C for 7 days. The islet cells were then cryopreserved using standard procedures. At specified intervals islets were thawed and cultured in M199, both in presence, and isolated from testes of male piglets according to a standard method.

20

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To test functional capacity, islets cultured for 3 and 7 days were assessed for insulin release in static incubation. In separate experiments, effect of insulin secretagogues was tested on islets cultured with and without Sertoli cells. The results of this study are shown in Tables 8 and 9.

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TABLE 8

Effect of insulin secretagogues, glucose and glucose plus Forskolin, on Insulin release from Incubated and Frozen/thawed (F/T) islets in the presence and absence of Pig Sertoli cells.

Insulin Release (uU/ml/10islets)

3.3mmol/L glucose 16.7 mmol/L glucose 16.7 mmol/L glucose
+ 100 umol Forskolin

Day 3 Incubated with Sertoli cells	42.3 ± 1.2	112.8 ± 17.7*#	267.7 ± 43.0**#
Day 3 Incubated alone	31.3 ± 2.1	57.3 ± 3.8*	123.4 ± 15.3**
Day 7 Incubated with Sertoli cells	22.9 ± 1.9	64.5 ± 6.4*#	153.9 ± 14.6**
Day 7 Incubated alone	21.3 ± 1.2	37.3 ± 6.0*	120.3 ± 11.4**
Day 3 F/T with Sertoli cells	20.6 ± 4.3	44.9 ± 9.9*	77.1 ± 13.7**
Day 3 F/T alone	11.7 ± 2.3	27.9 ± 6.6*	54.5 ± 10.7**

Anova Test: * vs 3.3mmol/L p 0.05, ** vs both 3.3 & 16.7 mmol/L P<0.05 # with sertoli cells vs islets alone P<0.05

TABLE 9

Effect of Sertoli cells on insulin content of incubated and frozen-thawed piglet islets.

	Insulin content (uU/10 islet(s))	
	Islets alone	Islets & Sertoli cells
Incubated D1	257.0 ± 19.6	391.1 ± 51.4~
Incubated D3	201.1 ± 19.1#	400.1 ± 41.0~#
Incubated D7	179.1 ± 26.2#	271.9 ± 39.9~#
Frozen D3/Thaw D3	52.4 ± 10.3	132.5 ± 35.1
Frozen D7/Thaw D3	10.4 ± 0.9	35.1 ± 8.2

Anova islets + Sertoli cell vs. islet alone P<0.05

Incubated islets D3, D7 vs. Frozen D3, D7 P<0.05

These results show that: (1) large numbers of neonatal porcine islets can be isolated by a simple method; (2)

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cryopreservation and thawing results in about 40% loss in number of islets in the absence of Sertoli cells and about a 20% loss in the presence of Sertoli cells; (3) cultured islets have the ability to respond to both glucose and glucose + Forskolin as insulin secretagogues; (4) the functional capacity of the co-cultured islet was enhanced two-fold in the presence of Sertoli cells;
 5 (5) following cryopreservation and thawing, islets recover more rapidly in presence of Sertoli cells and the response to both glucose and glucose + Forskolin was enhanced two fold in the presence of Sertoli cells.

Throughout this specification, unless the context requires otherwise, the word
 10 "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said method comprises administering xenografted Sertoli cells and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said xenografted Sertoli cells are administered in an amount effective to create an immunologically privileged site.
2. The method of claim 1 wherein said mammal is a human.
3. The method of claim 1 wherein said biological factor is a hormone.
4. The method of claim 1 wherein said biological factor is insulin and said disease is diabetes mellitus.
5. The method of claim 4 wherein said cells that produce said biological factor are pancreatic islet of Langerhans cells.
6. The method of claim 1 wherein said cells that produce said biological factor are cells transformed by a nucleic acid encoding said biological factor.
7. The method of claim 1 wherein said Sertoli cells are administered in a dosage ranging from 10^5 to 10^{10} cells.
8. The method of claim 1 wherein said cells that produce said biological factor are administered in a dosage of from 10^5 to 10^{10} cells.
9. The method of claim 1 which further comprises administering an immunosuppressive agent.

The method of claim 9 wherein said immunosuppressive agent is administered for a time



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sufficient to permit said transplanted cells to be functional.

11. The method of claim 9 wherein said immunosuppressive agent is cyclosporine.

12. The method of claim 11 wherein said cyclosporine is administered at a dosage of from 5 to 40 mg/kg body wt.

13. The method of claim 1 which further comprises administering a therapeutically effective amount of exogenous biological factor following the transplantation of said cells that produce said biological factor.

14. The method of claim 1 wherein said cells that produce said biological factor are co-cultured with Sertoli cells in tissue culture.

15. The method of claim 14 wherein said cells that produce said biological factor are cryopreserved prior to co-culturing with Sertoli cells in tissue culture.

16. A method of treating diabetes mellitus in a mammal wherein said method comprises administering xenografted Sertoli cells and a therapeutically effective amount of pancreatic islet of Langerhans cells to a diabetic mammal in need of such treatment wherein said xenografted Sertoli cells are administered in an amount effective to create an immunologically privileged site.

17. The method of claim 16 wherein said diabetes mellitus is type I or type II.

18. The method of claim 16 wherein said mammal is a human.

19. The method of claim 16 wherein said Sertoli cells are human, bovine or porcine.

20. The method of claim 16 wherein said pancreatic islet of Langerhans cells are human, bovine or porcine.



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21. The method of claim 16 wherein said administration is by injection into the renal subcapsular space.
22. The method of claim 16 wherein said administration is by injection into the subcutaneous facie.
23. The method of claim 16 wherein said Sertoli cells are administered at a dosage ranging from 10^5 to 10^{10} cells.
24. The method of claim 16 wherein said islet of Langerhans cells are administered at a dosage ranging from 5-1000 islet cells/g body wt.
25. The method of claim 16 which further comprises the administration of an immunosuppressive agent.
26. The method of claim 16 wherein said immunosuppressive agent is administered for a time sufficient to permit the transplanted islets to be functional.
27. The method of claim 25 wherein said immunosuppressive agent is cyclosporine.
28. The method of claim 27 wherein said cyclosporine is administered at a dosage of 5 to 40 mg/kg body wt.
29. The method of claim 16 which further comprises administering a therapeutically effective amount of insulin following transplantation of said pancreatic islet of Langerhans cells.
30. A method of creating an immunologically privileged site in a mammal wherein said method comprises transplanting isolated xenografted Sertoli cells into a mammal.



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31. The method of claim 30 wherein said mammal is a human.
32. The method of claim 30 wherein said Sertoli cells are injected into the renal subcapsular space.
33. The method of claim 30 wherein said Sertoli cells are injected into the subcutaneous facie.
34. The method of claim 30 wherein said Sertoli cells are transplanted at a dosage ranging from 10^5 to 10^{10} cells.
35. The method of claim 30 wherein said Sertoli cells are human, bovine or porcine.
36. A method of increasing the recovery and proliferation of *ex vivo* biological factor producing cells comprising co-culturing said cells with Sertoli cells under conditions sufficient to achieve said increased recovery and proliferation.
37. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor and a pharmaceutically acceptable carrier.
38. The composition of claim 37 wherein said biological factor is a hormone.
39. The composition of claim 37 wherein said cells that produce a biological factor are pancreatic islet of Langerhans cells.
40. The composition of claim 37 wherein said cells that produce said biological factor are cells that are transformed by a nucleic acid encoding said biological factor.
41. A pharmaceutical composition comprising Sertoli cells, pancreatic islet of Langerhans cells and a pharmaceutically acceptable carrier.



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42. A pharmaceutical composition comprising Sertoli cells and a pharmaceutically acceptable carrier when used in accordance with the method of any one of claims 1 to 36.

43. A compartmentalized kit adapted to receive a first container adapted to contain Sertoli cells and a second container adapted to contain cells that produce a biological factor that is absent or defective in a disease when used in accordance with the method of any one of claims 1 to 36.

44. A compartmentalized kit adapted to receive a first container adapted to contain Sertoli cells and a second container adapted to contain pancreatic islet of Langerhans cells when used in accordance with the method of any one of claims 1 to 36.

45. An article of manufacture comprising a packaging material and Sertoli cells contained within said packaging material, wherein said Sertoli cells are effective for creating an immunologically privileged site in a mammal, and wherein said packaging material contains a label that indicates that said Sertoli cells can be used for creating an immunologically privileged site in a mammal, said article of manufacture when used in accordance with the method of any one of claims 1 to 30.

46. A method according to any one of claims 1 to 36 or a pharmaceutical composition according to any one of claims 37 to 42 or a kit according to claim 43 or 44 or an article according to claim 45 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this 17th day of November, 1999

Research Corporation Technologies, Inc.

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants



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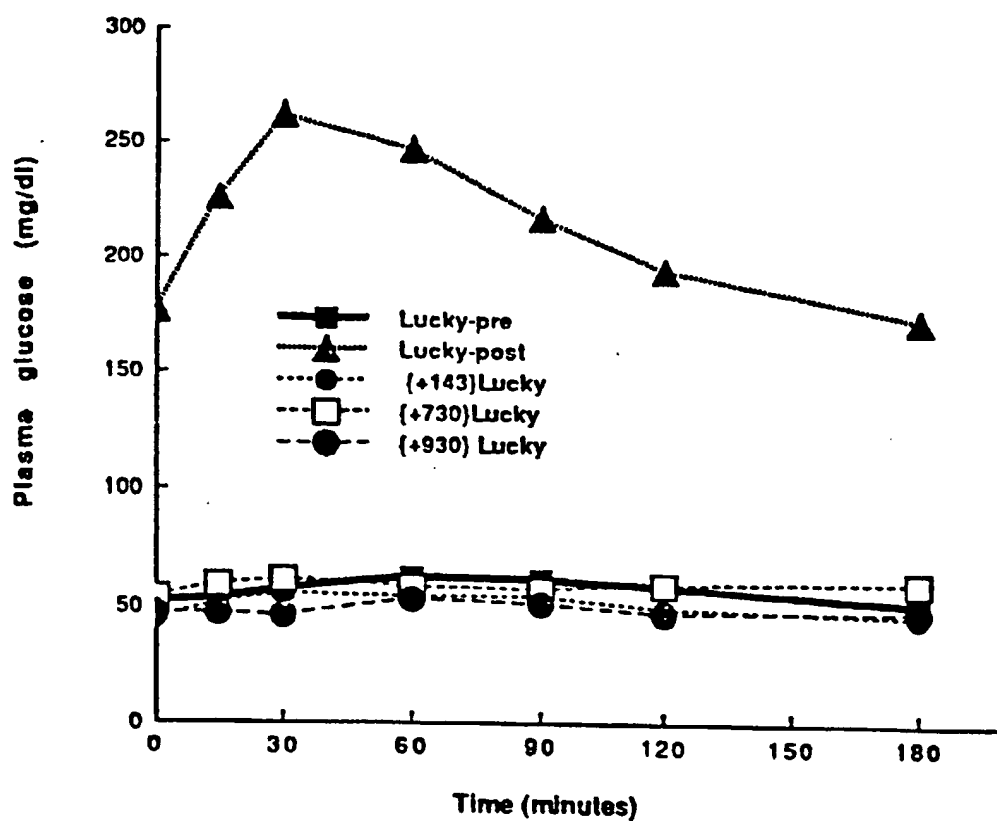


FIGURE 1

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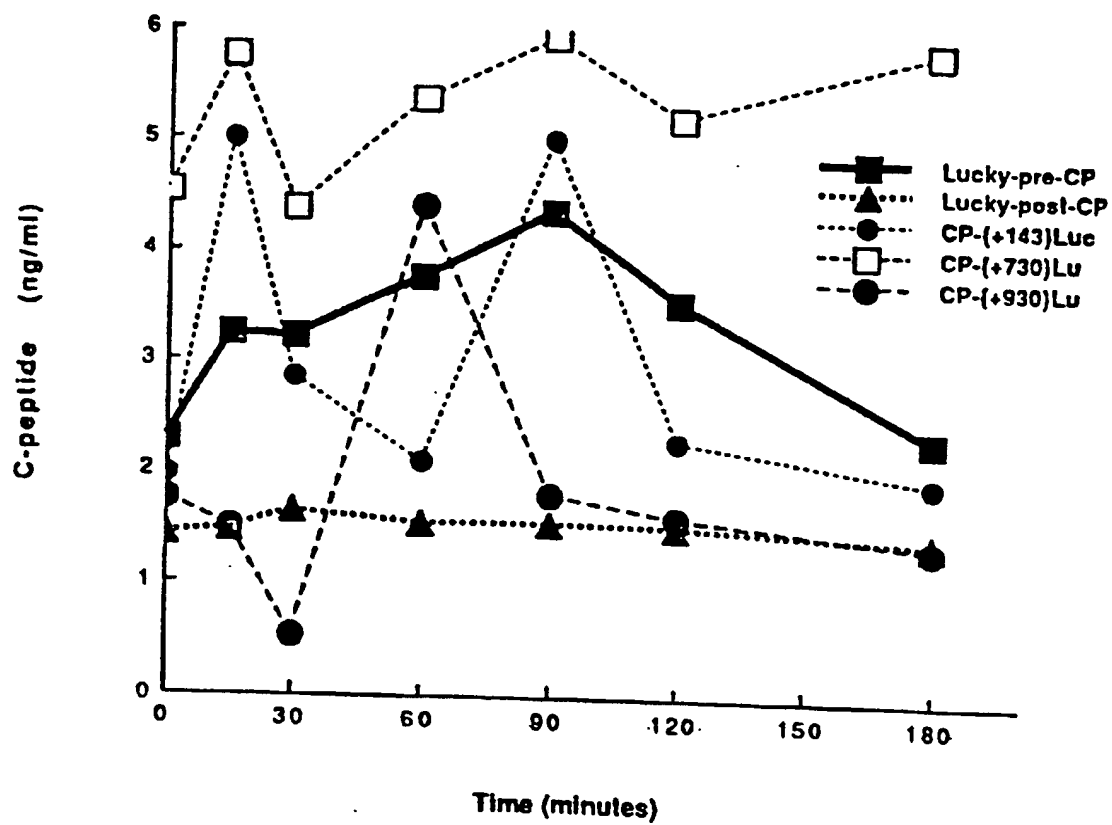


FIGURE 2

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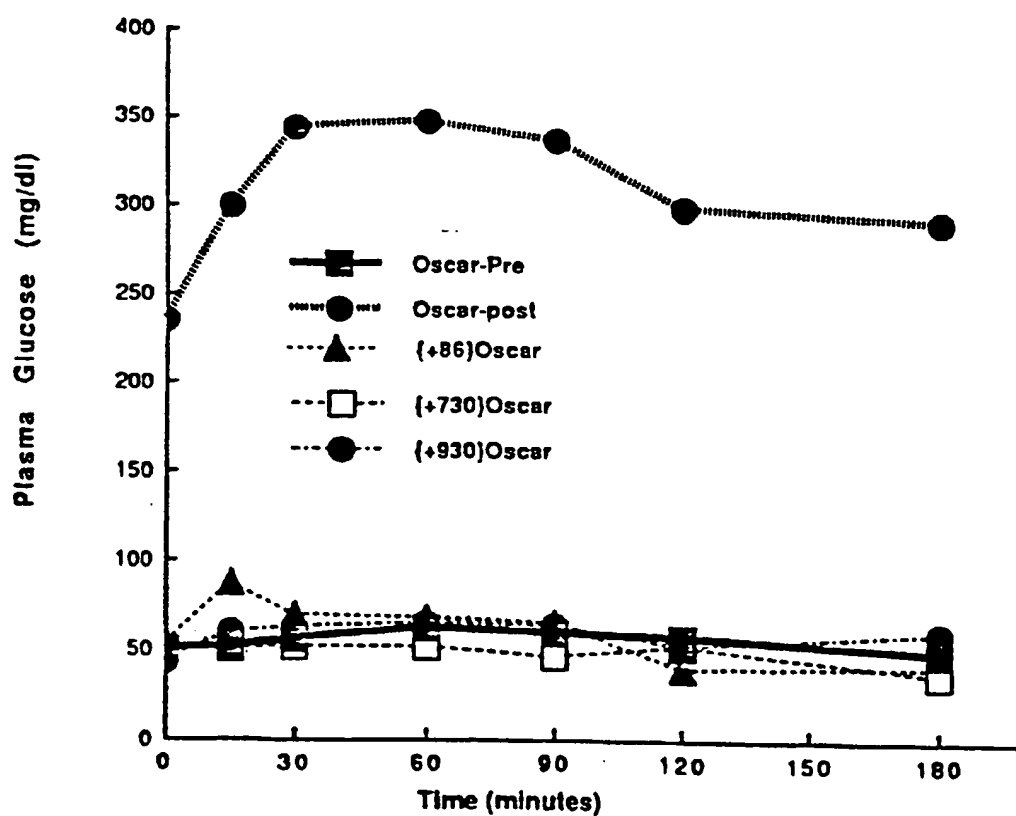


FIGURE 3

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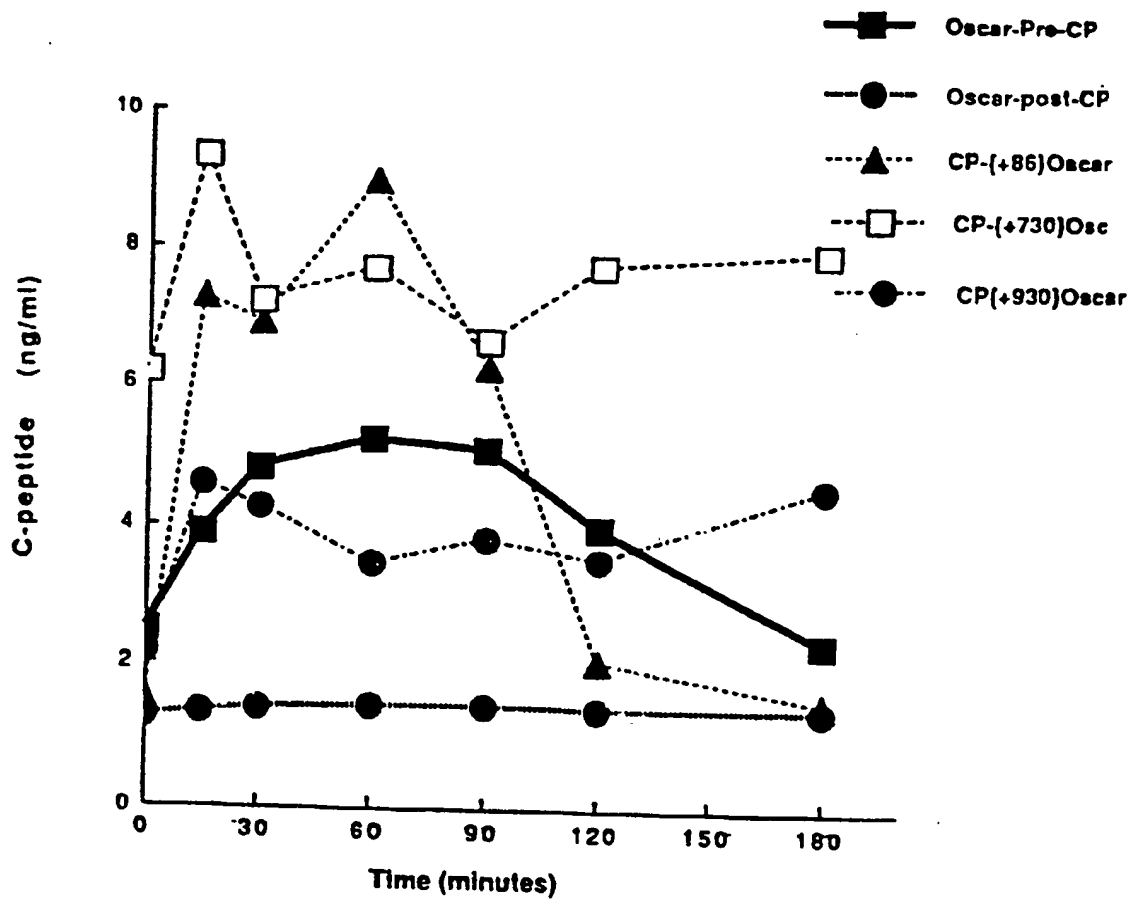
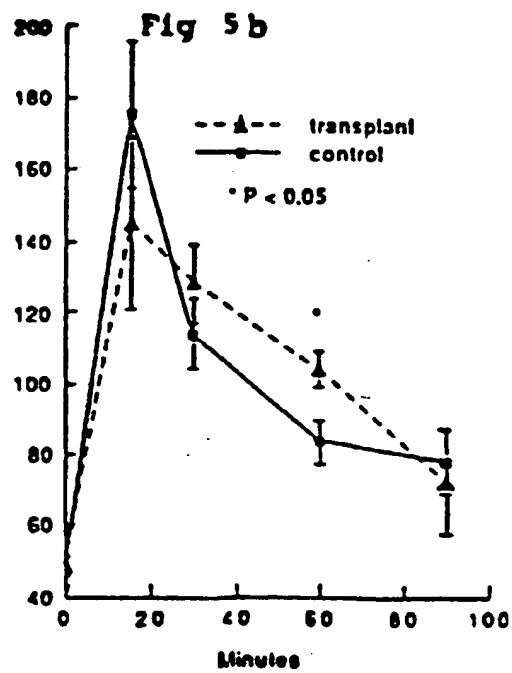
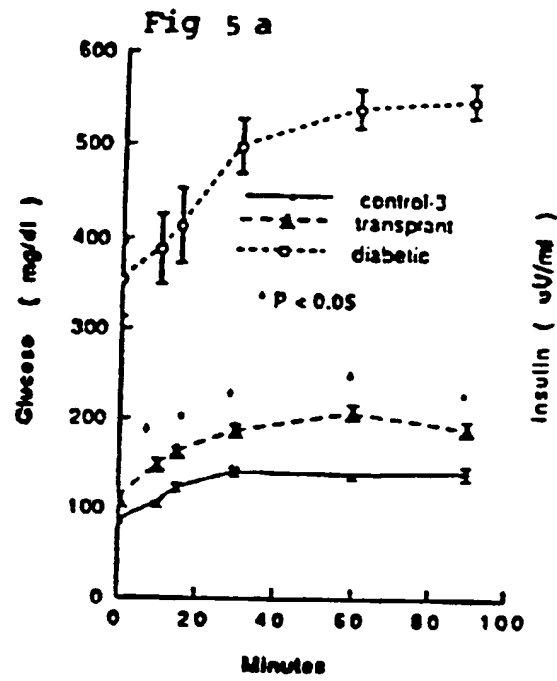


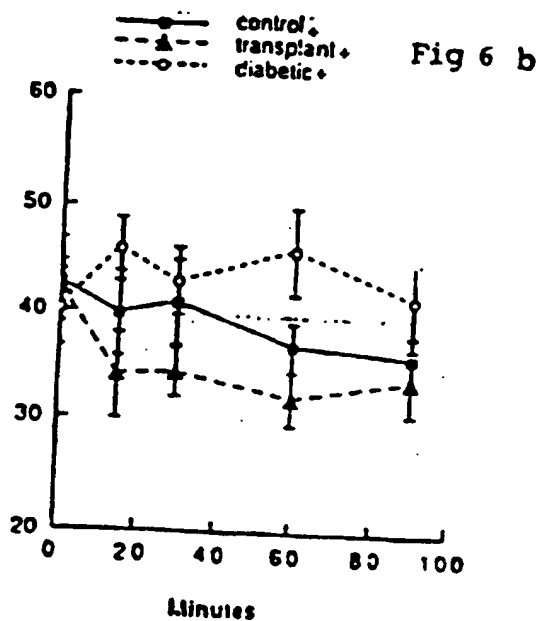
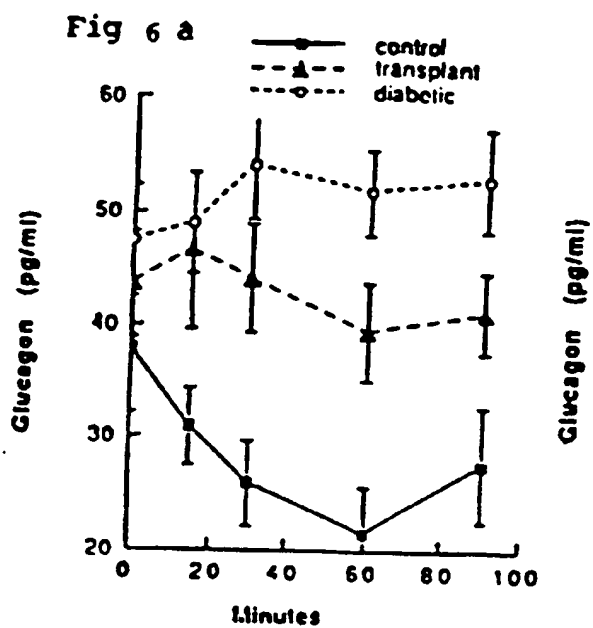
FIGURE 4

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**FIGURES 5a and 5b**

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**FIGURES 6a and 6b**

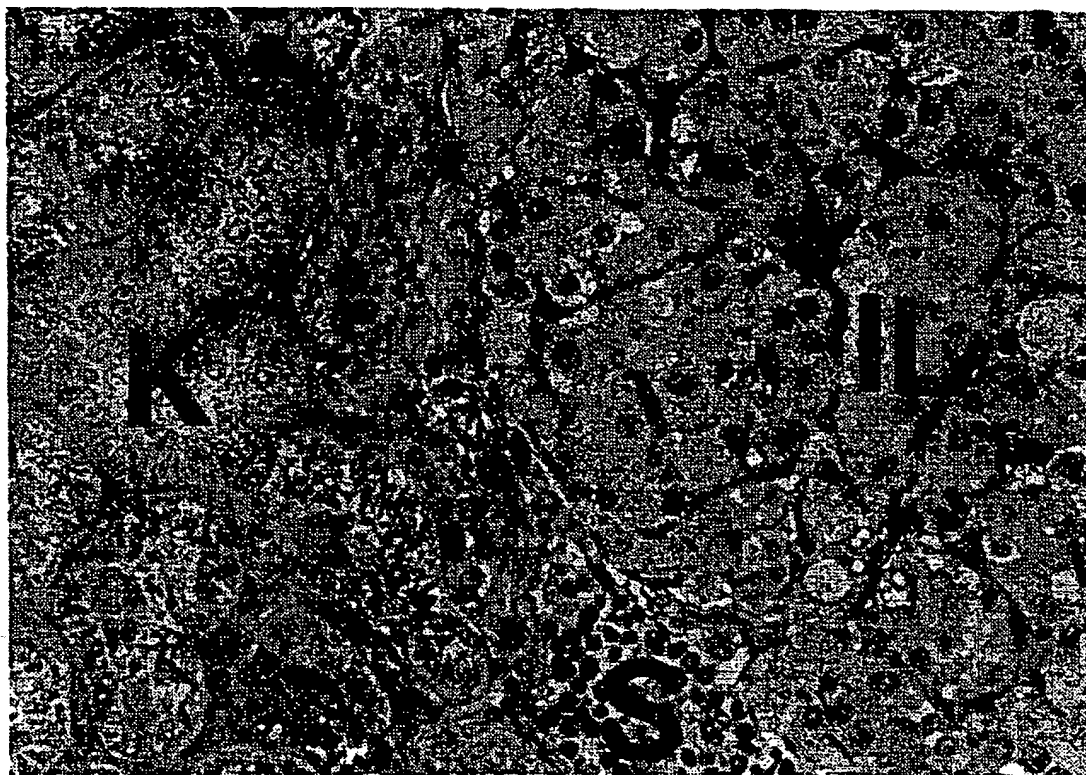


FIG. 7

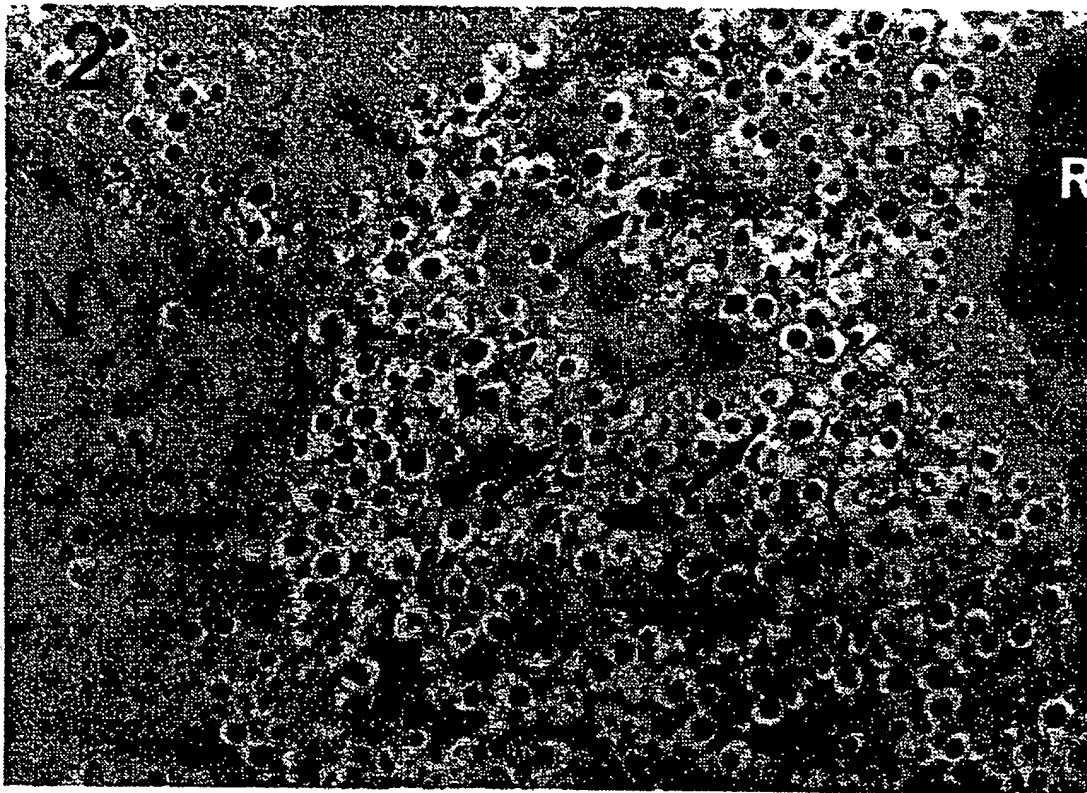


FIG. 8

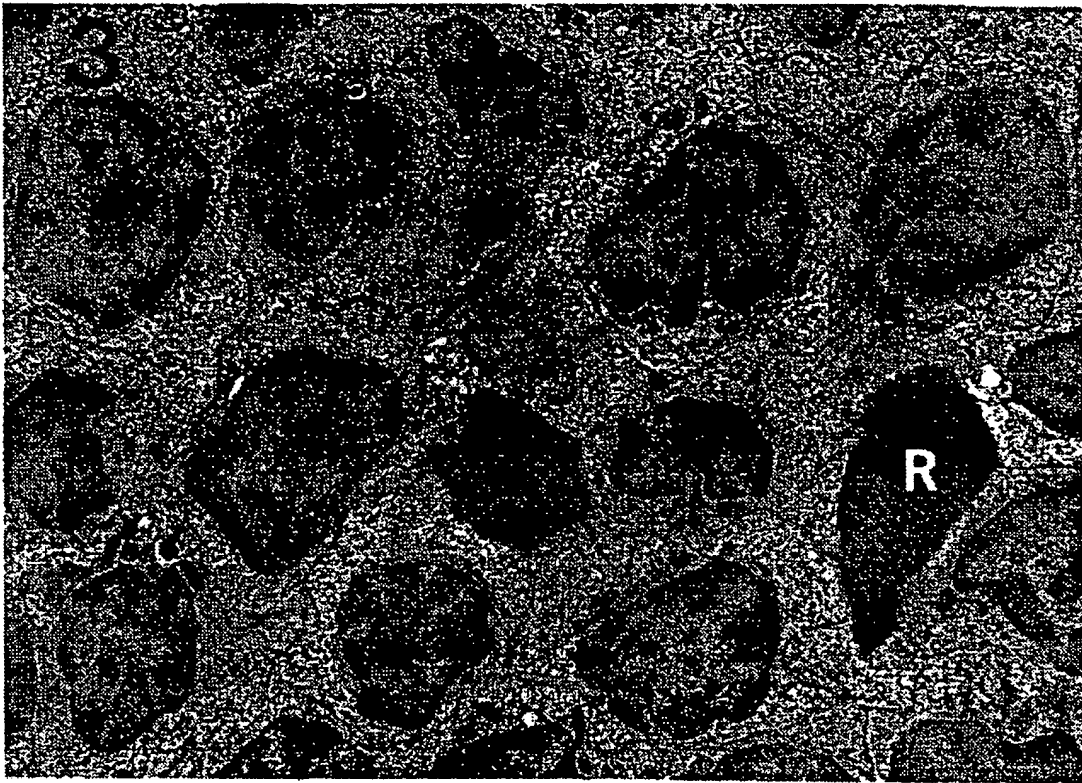


FIG. 9

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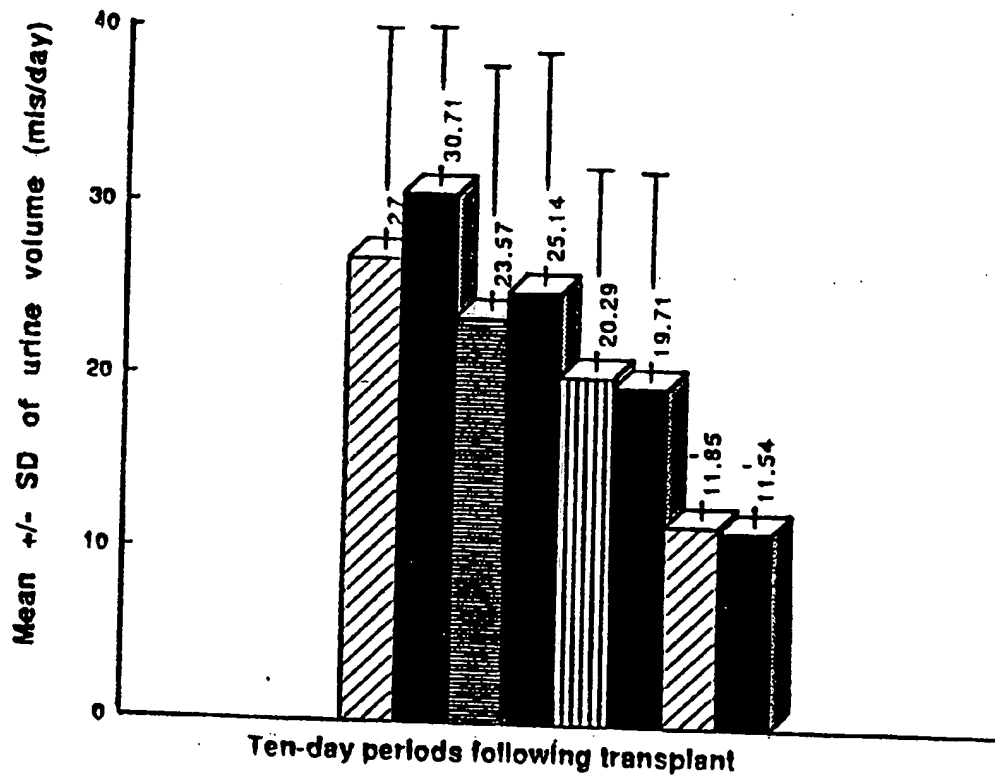


FIGURE 10

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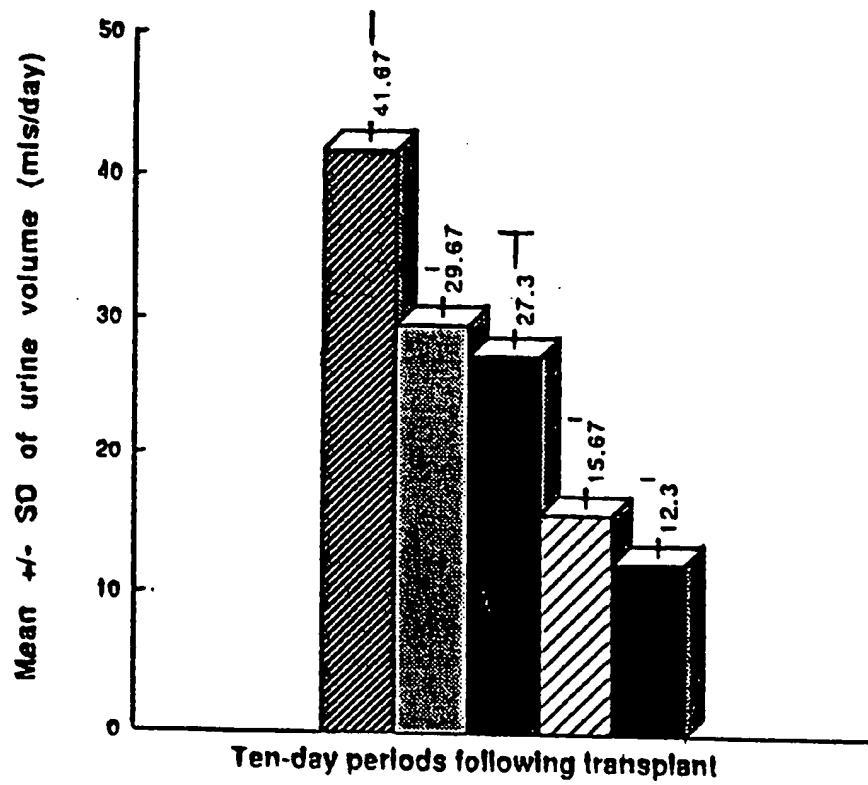


FIGURE 11

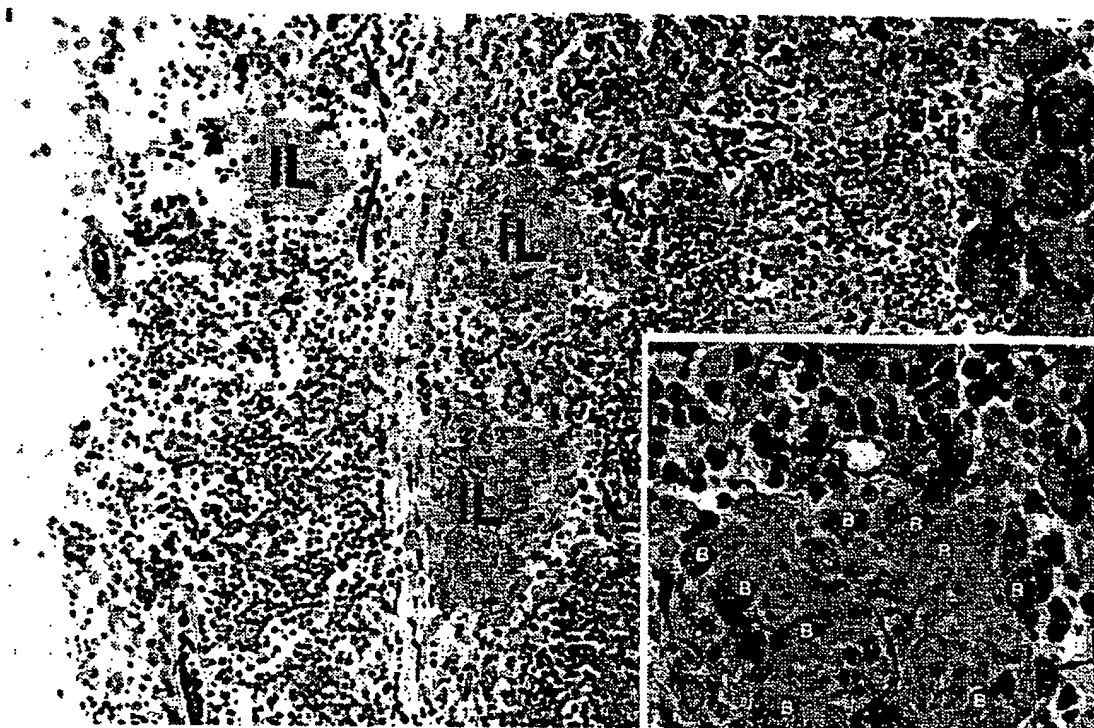


FIG. 12

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